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Potential Tumor Suppressor Function in Breast Cancer

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FOREWORD

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Introduction:

The E2F transcription factor plays a critical role in directly inducing the expression of genes that are necessary for a cell cycle progression into S phase. During times of cellular quiescence when expression of S phase genes would be inappropriate, the retinoblastoma protein, Rb, is bound to E2F forming a complex that promotes active repression of E2F target genes. Mutations in the Rb protein or in the cyclin dependent kinase inhibitor p16 which regulates the activity of Rb, are common abnormalities in human breast cancer. As key regulators of E2F and the cell growth control pathway, a thorough understanding of the functions of these proteins and the pathways they regulate will undoubtedly benefit the diagnosis and treatment of breast cancer. Our research therefore aims to elucidate the mechanism by which Rb and family member protein, p130, convert E2F from a positive acting factor into a negative acting complex.

Body:

Objective 1: To identify the mechanism of p130 repression

A plasmid expressing histone deacetylase was generated and transfected into C33A human cell lines along with p130 or Rb expression plasmids. Using co-immunoprecipitation, we were able to show that in fact Rb and p130 are capable of interacting with histone deacetylase in this system. In addition, a mutation of Rb that was originally found in a human tumor, Rb C706F, that has been shown to be ineffective as a transcriptional repressor, was incapable of interacting with histone deacetylase. This therefore defines a correlation between the ability of Rb to repress transcription and its ability to interact with histone deacetylase. Interestingly, using a yeast two hybrid assay, we were unable to show that Rb and histone deacetylase interact directly.

Having established that histone deacetylase likely played a role in mediating transcriptional repression through Rb, we then set out to determine if inhibitors of histone deacetylase could effect Rb mediated repression. Using a constitutively active SV40 promoter that contained upstream tandem Gal4 DNA binding domain sites, we assayed the ability of Gal4 DNA binding domain fusion proteins of Rb and p130 to repress this promoter. We found that in fact Rb and p130 were both capable of repressing transcription of this promoter. We reasoned that if repression of this promoter was due to recruitment of histone deacetylase, then inactivation of histone deacetylase by the specific inhibitor, TSA, should relieve this repression. Interestingly, when TSA was added to cells transfected with the reporter and the Gal4 fusions, no relief of repression was observed indicating that a histone deacetylase insensitive mechanism was involved in repression.

In addition to the examination of histone deacetylase as a potential mediator of transcriptional repression, we also examined proteins that we found in a yeast two hybrid assay to interact with the p130 protein. One protein, CtIP, was interesting in that it contains an LXCXE motif, a motif previously identified as being necessary for interaction

with the pocket domain of Rb family proteins. As the pocket domain of Rb family proteins is the domain implicated in mediating transcriptional repression, we examined this protein further for its potential to mediate repression through p130. We found that CtIP like histone deacetylase can interact with both p130 and Rb in co-immunoprecipitation experiments. In addition, the same transcriptional repression defective mutant, Rb C706F, that was found not to interact with histone deacetylase also is incapable of interacting with CtIP. This again correlates the ability of Rb and p130 to repress with their ability to interact with CtIP.

While we were investigating CtIP, it was published that CtIP interacts with a protein called CtBP and that CtBP has been shown in drosophila to act as a co-repressor. We hypothesized that CtIP could also act as a co-repressor by recruiting CtBP. In fact a Gal4DNA binding domain fusion protein of CtIP was capable of repressing the SV40 promoter and mutation of the domain of CtIP that is important for its interaction with CtBP decreases its ability to repress.

We therefore were able to conclude that p130 is able to mediated repression both by the recruitment of histone deacetylase and by the recruitment of a CtIP/CtBP complex. Potentially, the reason that TSA did not relieve repression of the SV40 promoter by Rb or p130 could be due to this second method of transcriptional repression which is likely histone deacetylase independent. We additionally speculate that there may in fact be even more mechanisms at work in concert with the two we have proposed. Our continuing studies involve the elucidation of the mechanism of repression of the CtIP/CtBP complex and the role of this complex in repressing endogenous E2F promoters.

The importance of this complex in human cancer and particularly breast cancer has very recently come to light. Experiments of others show that many human tumor lines over express the CtIP mRNA. In addition, the CtIP protein was recently shown to interact with the BRCA1 protein. Interestingly, this interaction is abolished in several tumor-associated

mutations of BRCA1 affecting the BRCT motifs indicating that in addition to mediating the activity of Rb, CtIP could also be important for tumor suppression by BRCA1.

Objective 2: To identify a region of p130 responsible for p130 mediated repression.

While this objective remains viable and interesting to us, the success of objective 1 has opened new avenues of research that we have been eager to address. Unfortunately, time constraints have prevented us from obtaining any data about this objective.

Objective3: To investigate the possibility that E2F4 is mutated in human breast carcinomas.

Many breast cancer cell lines were obtained from ATCC. Many of these lines were analyzed for the expression of E2F4, however, none tested were found to lack expression or express mutant forms of the protein. As the MD fellow that guided this objective, Dr. Paul Kelly Marcom, has left the lab to take a position in the Oncology department at Duke Medical Center, no further examination of this objective has been undertaken.

Appendices:

Key research accomplishments:

- 1 We established that Rb and p130 can immunoprecipitate histone deacetylase indicating that the proteins interact.
- 2 The histone deacetylase inhibitor, TSA, is incapable of derepressing most E2F target genes thereby establishing the likelihood of a histone deacetylase independent mechanism of transcriptional repression.
- 3 We identified a protein, CtIP, that interacts with p130 in a two-hybrid screen and by co-immunoprecipitation.
- 4 We characterized CtIP as having the activity of a transcriptional co-repressor.
- 5 We showed that CtIP repressed transcription, in part, through the CtBP protein.

List of reportable outcomes:

A manuscript "A novel mechanism for Rb/p130-mediated transcriptional repression involving recruitment of the CtBP co-repressor" is now in press at PNAS.

A poster was presented at the 1999 Cancer and the Cell Cycle meeting in Lausanne, Switzerland entitled, "The Rb and p130 proteins recruit CtIP/CtBP complexes to repress transcription."

PB-78

THE RB AND P130 PROTEINS RECRUIT CTIP/CTBP COMPLEXES TO REPRESS TRANSCRIPTION

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Previous work has demonstrated the critical role for E2F-dependent transcriptional repression in quiescent cells through the interaction with the retinoblastoma protein (Rb) and the Rb related protein p130. Although recruitment of histone deacetylase reflects at least part of the E2F/Rb-mediated repression, it is likely that additional events contribute as well. To further explore the transcriptional repression event, we used a yeast two hybrid screen to identify proteins that specifically interact with the p130 protein. From this screen, we identified a protein termed CtIP that interacts with p130 and Rb, dependent on an LXCXE motif within CtIP. CtIP has recently been characterized as a protein that interacts with the protein CtBP, a factor previously shown to interact with the C terminal sequences of adenovirus E1A. Recent work has demonstrated that the *Drosophila* homolog of CtBP, which shows 60% overall identity to the human CtBP protein, is a transcriptional co-repressor for the *Drosophila* repressor proteins *hairy*, *knirps*, and *snail*. Using Gal4 fusions in transient transfection assays, we show that either CtIP or CtBP is efficient in repressing an otherwise active SV40 promoter. The ability of Rb/p130 to repress transcription through CtIP is dependent on two distinct domains in CtIP: the LXCXE motif involved in Rb/p130 interaction as well as a PLDLS motif that coincides with sequence in *hairy*, *knirps*, and *snail* that has been shown to be important for interaction with dCtBP. We thus conclude that in addition to the ability of Rb and p130 to recruit histone deacetylase as a mechanism for transcriptional repression, the recruitment of the CtBP co-repressor, through an interaction with CtIP, represents an additional, distinct mechanism for E2F-mediated repression.

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**A Novel Mechanism for Rb/p130-Mediated Transcription Repression Involving
Recruitment of the CtBP Co-Repressor**

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ABSTRACT

Previous work has demonstrated the critical role for transcription repression in quiescent cells through the action of E2F-Rb or E2F-p130 complexes. Recent studies have shown that at least one mechanism for this repression involves the recruitment of histone deacetylase. Nevertheless, these studies also suggest that other events likely contribute to E2F/Rb-mediated repression. Using a yeast two hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we demonstrate that p130, as well as Rb, interacts with a protein known as CtIP. This interaction is dependent on the p130 pocket domain, that is important for repression activity, as well as an LXCXE sequence within CtIP, a motif previously shown to mediate interactions of viral proteins with Rb. CtIP interacts with CtBP, a protein named for its ability to interact with the C terminal sequences of adenovirus E1A. Recent work has demonstrated that the *Drosophila* homologue of CtBP is a transcriptional co-repressor for Hairy, Knirps, and Snail. We now show that both CtIP and CtBP can efficiently repress transcription when recruited to a promoter by the Gal4 DNA binding domain, thereby identifying them as co-repressor proteins. Moreover, the full repression activity of CtIP requires a PLDLS domain that is also necessary for the interaction with CtBP. We propose that E2F-mediated repression involves at least two events, either the recruitment of a histone deacetylase or the recruitment of the CtIP/CtBP co-repressor complex.

INTRODUCTION

The control of the early events of cell proliferation through the action of the G1 cyclin-dependent kinases (cdk), leading to the phosphorylation of Rb and related proteins, and the subsequent accumulation of E2F transcription factor activity is now well established [for reviews, see (1-6)]. It is also evident that most if not all human cancers arise as a result of the disruption of this pathway, either through the activation of positive acting components such as the G1 cyclins, or the inactivation of negative-acting components such as p53, Rb, and the cyclin kinase inhibitors (6, 7).

E2F transcription activity is now recognized to be a complex array of DNA binding activities that function both as transcriptional activating proteins as well as transcription repressors (8, 9). The E2F4 and E2F5 proteins, that specifically associate with the Rb related p130 protein in quiescent cells (10), function to repress transcription of various genes encoding proteins important for cell growth. In contrast, the E2F1, E2F2, and E2F3 proteins are tightly regulated by cell proliferation, accumulate as cells progress through mid to late G1, and appear to function as positive regulators of transcription. The complexity of E2F transcription control is illustrated by the fact that the E2F1, E2F2, and E2F3 genes are repressed in quiescent cells through the action of E2F4 or E2F5 complexes containing Rb or p130. In addition to the E2F1, E2F2, and E2F3 genes, the targets for E2F-mediated repression include a very large number of genes that encode proteins that guide cell cycle progression and that participate directly in DNA replication (8, 9).

Initial studies of Dean and colleagues clearly demonstrated that the role of Rb in controlling E2F-dependent transcription was not merely an inhibition of positive activation of transcription but rather, that E2F-Rb mediated repression was a dominant event, capable of

shutting off an otherwise active promoter (11, 12). Indeed, a series of recent reports has provided evidence that one mechanism for this Rb-mediated repression involves an ability of Rb to recruit histone deacetylase to E2F-site containing promoters, presumably resulting in an alteration of chromatin conformation that hinders transcription (13-15).

Nevertheless, despite the evidence implicating histone deacetylase recruitment as a mechanism for Rb-mediated repression, several observations suggest that additional events may contribute to the repression. For example, many genes subject to E2F/Rb-mediated repression are not de-repressed by treatment with the histone deacetylase inhibitor trichostatin A (TSA) (13). Moreover, although the recruitment of histone deacetylase is effective in repressing some promoters, others appear to be unaffected. Based on these observations, it would appear that a histone deacetylase independent mechanism of transcriptional repression contributes to the Rb control of transcription.

To further explore the mechanistic basis for Rb-mediated repression, we have used a yeast two hybrid screen to identify proteins that specifically interact with the p130 protein. In so doing, we have identified a protein known as CtIP that interacts with p130 dependent on the p130 pocket domain. CtIP has previously been described (16) as a protein that interacts with CtBP, an adenovirus E1A-interacting protein (17), and CtBP has recently been shown to function as a co-repressor in *Drosophila*. We show here that the CtIP protein can itself repress transcription and that this repression is due, at least in part, to its ability to recruit the CtBP co-repressor. Rb/p130 mediated repression therefore functions not only through histone deacetylase activity but also through a CtIP/CtBP repressor complex.

MATERIALS and METHODS

Cell Culture. C33A cells were grown in DMEM containing 10% fetal bovine serum.

Plasmids and Reagents. The SV40 promoter containing upstream Gal4 sites (pSVECG) was a kind gift from D. Dean (12). The MLP with Gal4 sites was a kind gift from D. Dean and R. Eisenman, the Gal4-HDAC expression plasmid was a kind gift from D. Reinberg (18), and the Rb Δ p34 plasmid was a kind gift from R. Bremner (19). The Gal4Rb plasmids were created in several steps. The Pvu II Fragment of Rb was first subcloned into the Sma I site of the pGBT9 vector. The BsaH-Bgl II fragment of Rb was then cloned into the Cla I and Bgl II sites of the pSP70 cloning vector. An EcoR I fragment was isolated from Rb-pSP70 (including the upstream EcoRI site from the polylinker) and cloned into the Rb-pGBT9 vector creating a full length Gal4Rb-pGBT9 plasmid used for expression in yeast. A Hind III fragment was isolated from the Gal4Rb-pGBT9 plasmid and cloned into the same site in pCDNA3 to create the Gal4Rb-pCDNA3 plasmid used for expression in mammalian cells. The Gal4p130 plasmid was created by digesting Bluescript, which contained full length p130 in the Hind III site, with BamH I and Sal I. This fragment was then cloned into the BamH I and Sal I sites of the pGBT9 vector. To put the fragment in frame with the Gal4 DNA binding domain, the p130-pGBT9 plasmid was cut with Eag I and Sma I and religated, creating Gal4p130-pGBT9 that was used for expression in yeast. To create Gal4p130 for expression in mammalian cells, a HindIII fragment of Gal4p130-pGBT9, including the Gal4DBD, was cloned into the HindIII site of pCDNA3. The p130^{C894F} and Rb^{C706F} mutants were made using the Clontech Transformer Site-Directed Mutagenesis Kit and the primers: 5'

CAA ATT ATG ATG TTT TCC ATG TAT GG 3' for Rb and 5' CAG TTA TTA ATG TTT GCC ATT TAT GTG 3' for p130. The p130 pocket domain-containing plasmid was made by PCR using primers with BamH I sites followed by 5' CCA GTT TCT ACA GCT ACG CAT 3' and 5' TTA ATG TGG GGA AAT GTA GAC 3'. The BamH I fragment was cloned into the BamH I site of a pCDNA3-Gal4DBD plasmid. The pCDNA3-Gal4DBD plasmid was made by cloning the Hind III-Sal I fragment from pGBT9 into the Hind III and Xho I sites of pCDNA3. The Gal4AD CtIP clone was isolated from the two hybrid screen. Full length Gal4AD CtIP was created by PCR using the Gal4AD human fetal liver library as the template with the primers: 5' GTT ACT GTA ATA GAT ACA AA 3' and 5' AAA AGG GCC CCT ATG TCT TCT GCT CCT TGC 3'. The PCR product was cut with BsrG I and Apa I and subcloned into the same sites in Gal4AD CtIP. The resulting full length Gal4AD CtIP was sequenced to confirm that no mutations were introduced. Myc-CtIP was created by subcloning the Bgl II - Apa I fragment of Gal4AD CtIP into the BamH I and Apa I sites of the pCDNA3-Myc vector. The Myc-CtIP Δ LXCXE was created by cutting the Myc-CtIP vector with BamHI and Hpa I, treating with Klenow to fill the DNA ends, and then religating with DNA ligase. The Myc-CtIP Δ PLDLS vector was made in several steps. Using PCR with the primers 5' TTT AGC AAC ACT TGT 3' and 5' AAA AGG ATC CTT TAT CCA TCA CAC 3', an N terminal fragment of CtIP was made. This fragment was subcloned into Bluescript at the Xba I and BamH I sites. A second fragment was created by PCR using the primers 5' AAA AGG ATC CGA TCG ATT TTC AGC 3' and 5' AAA AGG GCC CCT ATG TCT TCT GCT CCT TGC 3'. This fragment was subcloned behind the first fragment in the Bluescript BamH I and Apa I sites. The whole fragment of CtIP, that now contained a deletion of the PLDLS motif, was cut out of Bluescript using Xba I and Apa I and subcloned into the same

sites of the Myc-CtIP vector. Gal4BD CtIP was made by subcloning the BamH I - Apa I fragment from Myc-CtIP into the Bgl II and Apa I sites of the pCDNA3 Gal4DBD plasmid. Gal4BD CtIP Δ PLDLS was generated by subcloning the BamH I - Apa I fragment from Myc-CtIP Δ PLDLS into the Bgl II and Apa I sites of the pCDNA3 Gal4DBD plasmid. Gal4BD CtBP was made by PCR using the primers 5' AAA AGA ATT CAT GGG CAG CTC GCA CTT GCT 3' and 5' AAA ATC TAG ACT ACA ACT GGT CAC TGG CGT 3' and employing the CtBP clone that was a kind gift from G. Chinnadurai as a template. The PCR product was digested with EcoR I and Xba I and cloned into the same sites of the pCDNA3 Gal4DBD plasmid.

Repression Assays. C33A cells were transiently transfected by the calcium phosphate method with the pSVECG reporter and Gal4DBD fusion proteins. One μ g of the β -gal plasmid was co-transfected as a control for transfection efficiency. After 15 hours, cells were washed twice with PBS and allowed to recover in DMEM with 10% serum. Forty hours post transfection, cells were harvested and CAT assays performed as described previously (20) although extracts were not heat inactivated. CAT assay reaction mixture included 75 μ l extract, 75 μ l 1M Tris.Cl (7.8) 1 μ l C^{14} -labeled chloramphenicol (1mCi/ml), and 30 μ l of acetyl coenzyme A (3.5 mg/ml in H_2O). Reactions were incubated at 37° for 3.5 hours. CAT values were normalized relative to the vector alone control β -gal values. β -Gal activity was measured by adding 10 μ l of the extract prepared for the CAT assays to 590 μ l of 0.1mg/ml CPRG in lac Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM $MgSO_4$, 38 mM 2-mercaptoethanol, pH 7.0). The absorbance of each sample was measured at 570 nm. In

all instances of comparison, westerns were performed to determine that equal protein was expressed.

Yeast Two-Hybrid Screen. The yeast two hybrid screen was performed as recommended in the Clontech protocol. Inserts from positive clones were sequenced according to Sequenase Kit (USB) instructions.

Immunoprecipitations. C33A cells were transiently transfected by the calcium phosphate method. After 15 hours of transfection, cells were washed twice with DMEM and then complete media was replaced. Forty hours post transfection, cells were harvested and lysed in Ip buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP40, and the protease inhibitors, Leupeptin 1 μ g/ml, Aprotinin 1 μ g/ml, Pepstatin 1 μ g/ml, PMSF or Perfablock (Boehringer Mannheim) at 1mM. Extracts were precleared by incubating with protein A agarose beads (Calbiochem) for 1 hour and then centrifuged at 15K RPM for 10 minutes. An aliquot of the sample (10%) was used as input and 10% was used in a β -gal assay. The amount of extract used in the immunoprecipitation was normalized based on the β -gal values. One μ g of the appropriate antibody was added to precleared extracts and allowed to mix at 4° for 3 hours. Protein A agarose beads were then added and allowed to mix at 4° for 1.5 hours. Samples were then washed 4 times at 4° with 1 ml of IP buffer and run on an SDS polyacrylamide gel.

RESULTS

Recent work has provided evidence for a mechanism for E2F/Rb-mediated repression that involves the recruitment of histone deacetylase (13-15). In particular, these studies demonstrated an ability of Rb to physically interact with HDAC that coincided with the ability of Rb to repress transcription. Nevertheless, this work also suggested that additional events may contribute to the ability of Rb to repress transcription. For instance, whereas the addition of the HDAC inhibitor, trichostatin A (TSA) reversed the repression of the adenovirus major late promoter, TSA had little effect on the ability of Rb to repress transcription of the SV40 or TK promoter (13). Moreover, although the MAD protein, which is known to repress transcription through the recruitment of HDAC (21), could efficiently repress the major late promoter, it had no effect on the SV40 promoter (13). Although the distinction between these promoters remains unclear, the apparent insensitivity of the SV40 promoter to the recruitment of HDAC provides an assay to examine mechanisms of Rb-mediated repression that are independent of HDAC recruitment.

To further explore the basis for HDAC-independent E2F/Rb-mediated repression, we assayed wildtype and mutant versions of the Rb family proteins for their ability to repress transcription driven by an SV40 promoter that also contained upstream Gal4 sites (Figure 1A). As shown in Figure 1B, fusion proteins linking either Rb or p130 to the Gal4 DNA binding domain were capable of repressing transcription of the reporter in C33A cells. Similarly, a mutant of Rb that deletes eight sites for phosphorylation by cdc2 (Rb Δ p34), is an even more efficient repressor of transcription than wildtype Rb (19). As previously published for Rb (12), the pocket domain of p130, when tethered to the Gal4 DNA binding domain, repressed transcription indicating that the pocket domain is sufficient for repression of the

SV40 promoter. An Rb mutation found in human tumors, involving a Cys to Phe change at position 706 within the pocket domain (Rb^{C706F}), has been shown to disrupt the structure of the pocket domain and therefore to abolish the interaction of Rb with the viral oncoproteins E1A and T antigen (22). As previously shown by Dean and colleagues (12), the Rb^{C706F} mutant failed to repress transcription of the SV40 promoter (Figure 1B). Likewise, a p130 mutant constructed to contain the equivalent alteration in the homologous sequence (p130^{C894F}) also failed to repress transcription (Figure 1B).

Rb and p130 Interact with CtIP

Given the indication from previous work that E2F/Rb-mediated repression could not be fully explained by recruitment of histone deacetylase, we initiated a search for other proteins that might be involved in a HDAC-independent transcriptional repression by Rb or p130. We used a full length p130 protein in a yeast two-hybrid assay to screen for potential protein partners of p130 that could mediate this HDAC-independent repression. The HF7C yeast strain was transformed with a plasmid encoding a Gal4 DNA binding domain-p130 fusion protein together with a human fetal liver cDNA library that incorporated the Gal4 activation domain. Forty positive transformants yielded 11 different clones encoding proteins that interacted with p130. Among the positive clones were cyclin D1, cyclin D3, E2F4, and E2F5, proteins known to specifically interact with Rb/p130.

In addition to these anticipated interacting proteins, one clone was found to encode the first 800 amino acids of a protein previously identified as CtIP. CtIP (C terminal interacting protein) was originally recovered in a yeast two-hybrid screen as a partner of a protein known as CtBP (C terminal binding protein) (16), a protein that binds to the C terminus of adenovirus

E1A (23). The amino terminal portion of CtIP contains an LXCXE sequence (Figure 2A), a motif found in the viral oncoproteins E1A, T antigen, and E7, as well as the D type cyclins, that mediates the interaction with the Rb family proteins. Given the presence of the LXCXE motif in CtIP, we tested the ability of CtIP to interact with Rb in the two hybrid assay. Figure 2B shows that CtIP can specifically interact with p130 and Rb but not with an unrelated yeast protein, KSS1.

We also used a co-immunoprecipitation assay to measure the ability of CtIP to interact with p130, as well as to define the sequences important in each protein for the interaction. As shown in Figure 2C, wild type p130 could be recovered in an immunoprecipitate with the CtIP protein but a pocket disrupting mutant p130 protein (p130^{C894F}) could not. In addition, p130 could be found to associate with the wild type CtIP but not with a mutant of CtIP in which the first 170 amino acids, including the LXCXE domain, was deleted (Figure 2D). It thus appears clear that CtIP interacts with p130, as well as Rb, and does so via an LXCXE-pocket domain interaction.

The observation that p130 interacts with CtIP, dependent on the pocket domain that is also required for p130-mediated repression, suggested a possible role for CtIP in transcriptional repression. To explore such a function, a fusion protein containing the Gal4 DNA binding domain linked to CtIP was created and assayed for its ability to repress the SV40 promoter reporter construct. As shown in Figure 3, the Gal4-CtIP fusion protein was indeed active as a repressor; in fact, the Gal4-CtIP protein was as efficient as the Gal4-p130 fusion protein in the repression of the SV40 promoter. Based on all of these results, we conclude that recruitment of the CtIP protein represents an alternative mechanism, in addition to the recruitment of histone deacetylase, for p130-mediated repression.

CtIP Recruits the CtBP Co-Repressor

CtIP was isolated based on its interaction with CtBP, a protein identified as an adenovirus E1A-binding protein (16). More recently, a *Drosophila* homologue of CtBP has been identified and shown to function as a co-repressor for the transcriptional regulatory proteins Hairy, Knirps, and Snail (24-26). These observations thus suggested the possibility that CtIP might function in E2F/p130-mediated repression by recruiting the CtBP protein. To investigate this possibility, we first examined the ability of CtIP to interact with CtBP. C33A cells were co-transfected with a plasmid encoding a Myc-tagged CtIP protein and a plasmid encoding Gal4-CtBP. Cells were then assayed for an interaction of the two proteins by immunoprecipitating Gal4-CtBP and then assaying for the presence of CtIP in the immunoprecipitates by Western blotting. As shown in Figure 4A, wildtype CtIP was indeed recovered in the CtBP immunoprecipitate.

Previous work has demonstrated that the interaction of CtBP with E1A, or the interaction of the *Drosophila* CtBP with Hairy, Knirps, and Snail, is dependent on a PLDLS sequence found within these interacting proteins. Examination of the CtIP sequence reveals a PLDLS motif within the C terminal region of the protein (see Figure 2A). As such, we have generated a deletion mutant lacking this sequence and have tested the ability of the mutant to interact with CtBP. As shown in Figure 4A, deletion of the PLDLS sequence in CtIP abolished the interaction with CtBP. We thus conclude that CtIP and CtBP do indeed interact and, like the interaction of the *Drosophila* proteins, the interaction is dependent on the PLDLS domain of CtIP.

Finally, to explore the role of CtBP in E2F/p130/CtIP-mediated repression, we assayed the effect of the CtIP PLDLS mutation on CtIP-mediated repression. As shown in Figure 4B, the repressing activity of CtIP was clearly impaired by the PLDLS mutation, coincident with the role of the PLDLS sequence in mediating the CtBP interaction. Given the indication that CtIP has the ability to repress transcription when recruited to a promoter, together with the evidence that CtIP can interact with CtBP, we assayed the ability of CtBP alone to function as a transcriptional repressor. A Gal4 DNA binding domain-CtBP fusion was assayed for its ability to repress the SV40 promoter. As shown in Figure 4C, the Gal4-CtBP fusion was equally effective as the Gal4-CtIP fusion protein in repressing the SV40 promoter.

Based on these results, we conclude that the CtBP protein does possess transcriptional repressing activity and that the recruitment of CtBP via an interaction with CtIP represents an alternate mechanism for E2F/Rb-mediated repression of transcription.

DISCUSSION

The role of histone deacetylase recruitment in transcription repression, including E2F/Rb-mediated transcription repression, has now been shown in multiple instances (13-15, 27). Nevertheless, it is also clear that other mechanisms, functioning independently of HDAC recruitment, must play a role in repression. The data we present here now describes at least one additional mechanism for E2F/Rb-mediated repression that involves the recruitment of the CtIP/CtBP co-repressor complex.

Alternate Mechanisms of E2F/Rb-Mediated Repression

The CtBP protein has been implicated in several forms of transcription repression including the recent studies of the *Drosophila* proteins Hairy, Knirps, and Snail (24-26). In each case, CtBP is recruited to a promoter through the interaction with a PLDLS-containing protein. The results we now present here demonstrate that the mammalian CtBP protein can be recruited to a target promoter through an interaction with CtIP, which in turn interacts with Rb or p130. While the majority of the PLDLS-containing proteins that are known to interact with CtBP are DNA binding proteins, including Hairy, Knirps, and Snail, there is no evidence to suggest that CtIP has intrinsic DNA binding activity. Rather, CtIP appears to act as a bridging protein, bringing the CtBP co-repressor to a promoter through the interaction with Rb or p130 and then E2F (Figure 5). The observation that CtIP contains distinct motifs that can mediate Rb/p130 binding (LXCXE) as well as CtBP binding (PLDLS) provides a mechanism by which CtIP could serve to bridge the two sets of proteins.

Although CtIP does recruit CtBP, and this can serve as a mechanism for transcriptional repression, it is also possible that other proteins interact with CtIP, possibly

leading to other events of transcriptional repression. In this regard, it is of interest to note that while the recruitment of CtBP coincides with repression by Hairy as well as BKLF, each of these proteins appears to interact with other factors to establish a more complete repression (26, 28). Possibly, the fact that the CtIP PLDLS mutation did not completely abolish repression might suggest that other activities of CtIP could contribute to full repression.

Yet to be determined is the precise mechanism by which CtBP might effect a repression of transcription. Although there has been one report suggesting an interaction of CtBP with histone deacetylase (29), this is unlikely to be the primary mechanism of CtBP-mediated repression given our observations and the observations of others that the SV40 promoter, shown to be repressed by CtBP, is relatively insensitive to histone deacetylase (13). An alternative possibility stems from recent observations that CtBP interacts with the human polycomb proteins (30). Polycomb proteins have been shown in *Drosophila* to be important in repression of certain homeotic genes. While the mechanisms of this repression are unclear, current models speculate that the PcG proteins can package regions of DNA into heterochromatin-like structures (30).

Multiple Roles for E1A in Affecting Cellular Transcription

Human CtBP was originally identified as a phosphoprotein that associates with the C-terminus of E1A (17). The C terminal E1A sequences that are involved in the CtBP interaction are well conserved among adenovirus serotypes implying a functional importance. Nevertheless, the analysis of function of these sequences has been somewhat confusing. Some experiments suggest that the C terminal E1A domain functions to suppress cell transformation in conjunction with an activated Ras protein (23). That is, mutation of the

E1A C terminus, which include the domain responsible for binding to CtBP, leads to enhanced oncogenicity in conjunction with Ras. In contrast, other experiments have provided evidence for a role for these sequences in the immortalizing function of E1A as well as to collaborate with adenovirus E1B in transformation (31). Although the basis for the apparent discrepancy in these results is unclear, the latter findings, indicating a requirement for the C terminal domain in E1A function in immortalization and transformation with E1B, is certainly consistent with the findings that this domain interacts with CtBP and thus would disrupt the formation of the E2F-p130-CtIP-CtBP repressor complex. It is interesting to note that both E1A and CtIP contain LXCXE and PLDLS motifs implying that the two proteins target the same factors, Rb and CtBP, and perhaps compete for their binding. In this way, E1A would be seen to disrupt transcriptional repression in three complementary fashions - either the inhibition of Rb family protein interaction with E2F, the inhibition of the interaction of Rb with the CtIP/CtBP complex, or the inhibition of the CtBP repressor with the E2F complex. Interestingly, two recent reports describe yet another mechanism for E1A action that involves a direct inhibition of histone acetyl transferase activity (32, 33). In addition to its ability to disrupt complexes involving the p300 protein, these two reports demonstrate that the direct interaction of E1A with either p300/CBP or PCAF leads to an inhibition of histone acetylase activity. As such, it appears that the E1A protein has evolved a series of distinct activities to affect transcription through an alteration of chromatin structure.

ACKNOWLEDGEMENTS

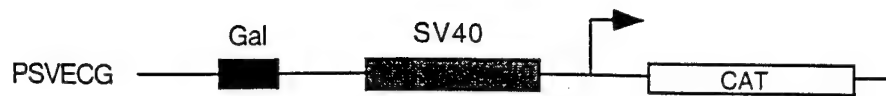
We are grateful to Jeanette Cook, Rosalie Sears, Chi-Hyun Park, and Nicole Liberatti for invaluable advice, discussion, and technical assistance. We are grateful to D. Dean, R. Eisenman, D. Reinberg, G. Chinnadurai, and R. Bremner for the generous gift of reagents. We also thank Kaye Culler for assistance in the preparation of the manuscript. This work was supported by the Howard Hughes Medical Institute. ARM was supported by a fellowship from the Department of the Army (DAMD17-98-8074) and JRN is an Investigator of the Howard Hughes Medical Institute.

Figure 1. Rb/p130-Mediated Repression Independent of Histone Deacetylase

Recruitment

- A. Schematic representation of the pSVECG reporter. Constitutive CAT expression is driven by the SV40 promoter/enhancer. Upstream Gal4 sites provide a binding site for Gal4 DNA binding domain fusion proteins.
- B. C33A cells were transiently transfected with 1 μ g β -gal, 0.5 μ g of the pSVECG reporter and 2 μ g of Gal4-Rb, Gal4-Rb Δ p34, Gal4-p130, or Gal4-p130^{C894F} or 5 μ g of Gal4-Rb^{C706F}. Western blotting was performed to verify that transfected constructs expressed equal protein. For the repression assay using the pocket domain, 0.5 μ g of pSVECG was transfected with 3 μ g of Gal4-p130 pocket. As controls, C33A cells were transfected with 1 μ g β -gal, 0.5 μ g pSVECG reporter and 2 or 3 μ g of a vector encoding a Gal4 DNA binding domain (control vector). Cells were harvested 40 hrs post-transfection and CAT activity was assayed. β -Gal values were used to normalize for transfection efficiency. Results of typical experiments are shown.

A.



B.

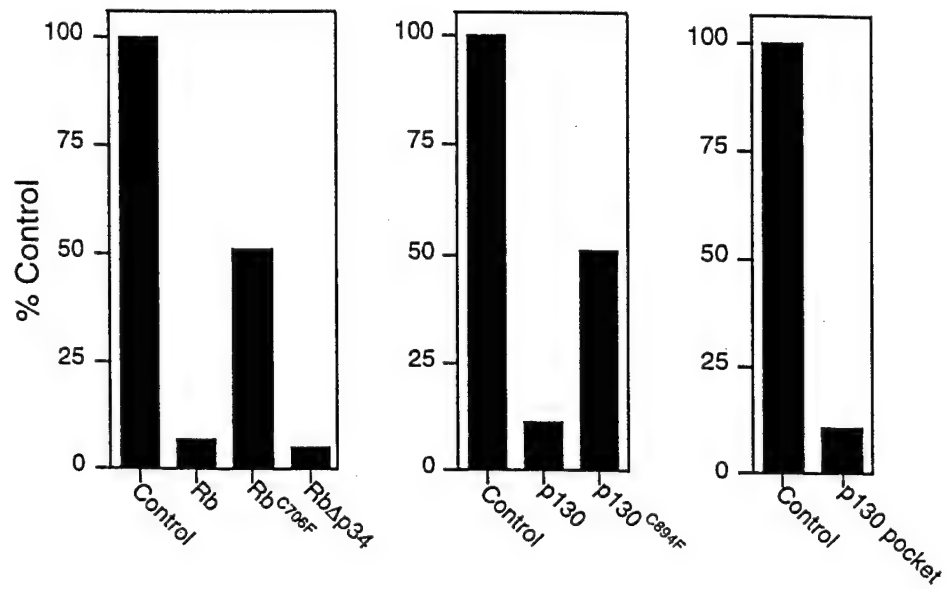


Figure 2. Rb/p130 Interaction with CtIP

- A. Schematic representation of the CtIP protein. The position of an LXCXE sequence motif and a PLDLS sequence motif within the 897 amino acid CtIP protein are indicated.
- B. HF7C yeast were transformed with plasmid encoding the Gal4AD-CtIP fusion protein alone or with a Gal4BD-p130, Gal4BD-Rb, Gal4BD-KSS1, or the empty Gal4BD vector. Yeast were streaked on non-selective media lacking Trp and Leu and on media that lacks Trp, Leu, and His that is selective for protein/protein interactions.
- C. C33A cells were transfected with 10 μ g of Myc-CtIP and 10 μ g of either Gal4-p130 or Gal4-p130^{C894F}. Cells were harvested 40 hrs post-transfection and lysed in IP buffer. Ten percent of the extract was loaded in input lanes 1-3. Myc antibody (Santa Cruz, 9E10) was used to immunoprecipitate Myc-CtIP. p130 antibody (Santa Cruz) was used in Western blotting to detect p130 in the immunoprecipitates (lanes 4-6). The blot was stripped and re-probed with Myc antibody to verify that equal amounts of Myc-CtIP were immunoprecipitated (lanes 4-6).
- D. C33A cells were transfected as in C with 10 μ g of Gal4-p130 and 10 μ g of either Myc-CtIP or Myc-CtIP Δ LXCXE. Ten percent of the extract was loaded in input lanes 1 and 2. Myc antibody was used to immunoprecipitate Myc-CtIP. p130 antibody was used in Western blotting to detect p130 in the immunoprecipitates (lanes 3 and 4). The blot was

stripped and re-probed with Myc antibody to verify that equal amounts of Myc-CtIP were immunoprecipitated (lanes 3 and 4).

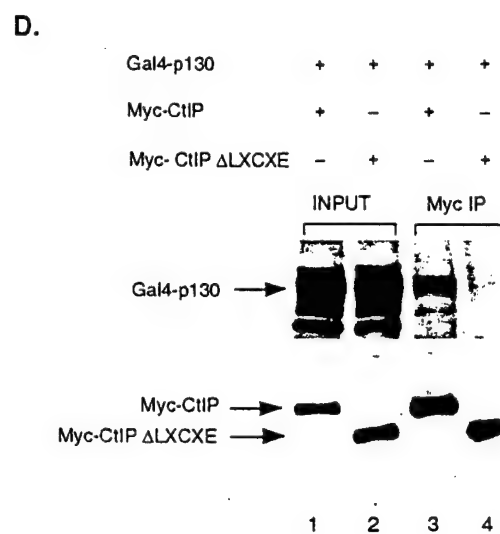
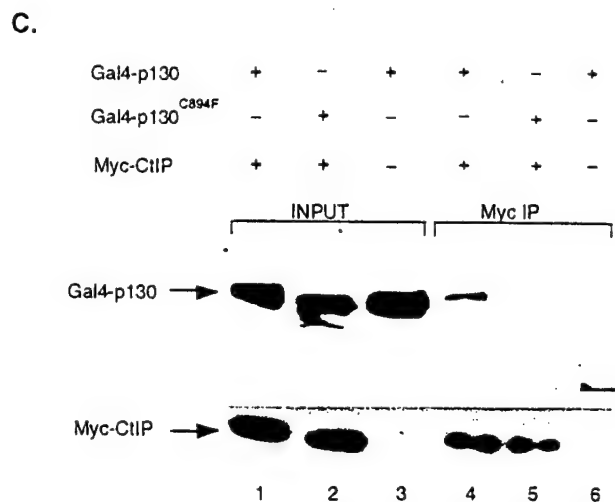
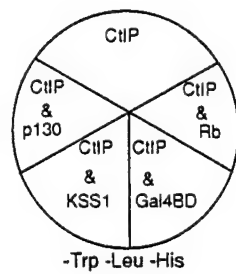
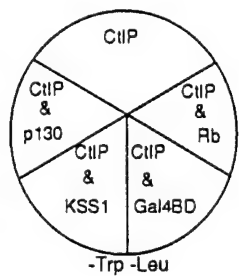
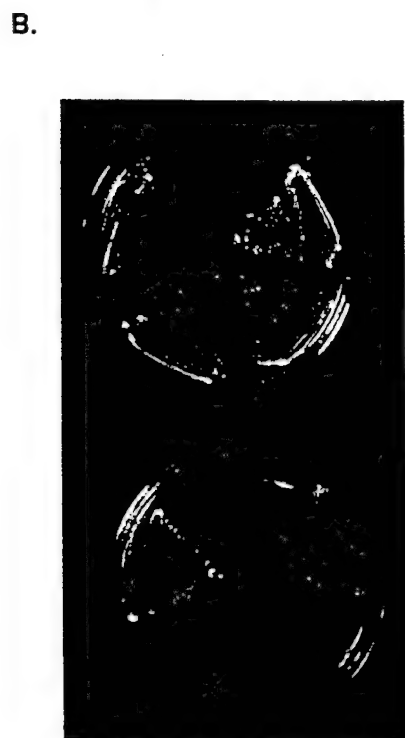
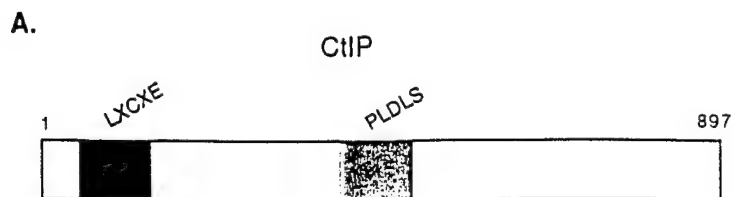


Figure 3. CtIP Is a Transcriptional Repressor

C33A cells were transiently transfected with 1 μ g β -gal, 0.5 μ g of the pSVECG reporter and 2 μ g of Gal4-p130 or 20 μ g of Gal4-CtIP. Western blotting was performed to verify that transfected constructs expressed equal protein. As controls, C33A cells were transfected with 1 μ g β -gal, 0.5 μ g pSVECG reporter and 2 μ g of a vector encoding a Gal4 DNA binding domain (control vector). Cells were harvested 40 hrs post-transfection and CAT activity was assayed. β -Gal values were used to normalize for transfection efficiency. Results of a typical experiment are shown.

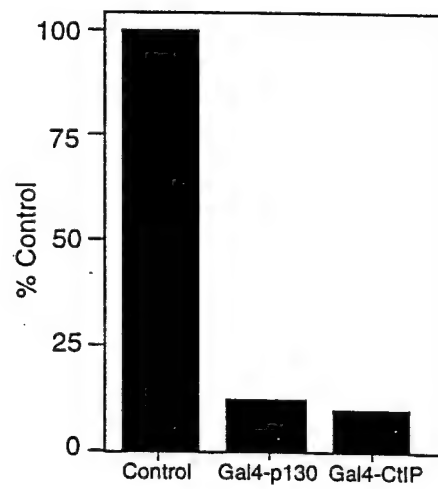
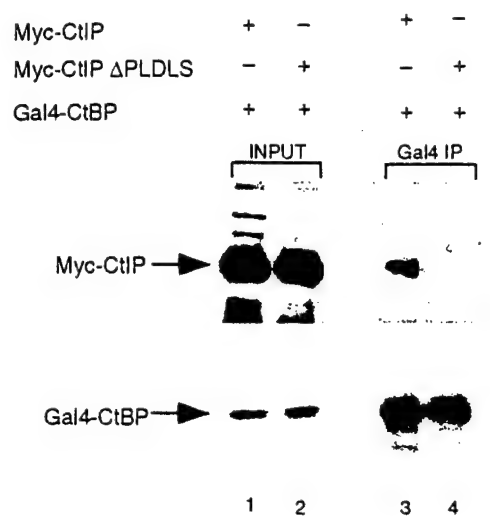


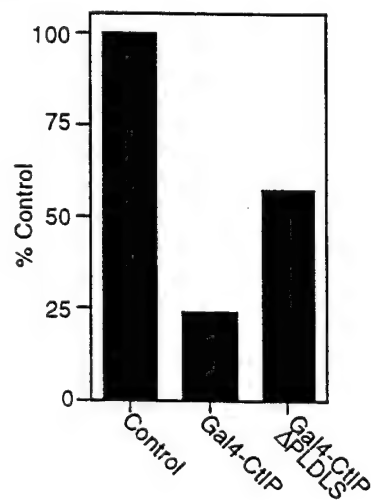
Figure 4. p130/CtIP-Mediated Repression Involves the Recruitment of the CtBP Co-repressor.

- A. C33A cells were transfected with 10 μ g of Gal4-CtBP and 10 μ g of either Myc-CtIP or Myc-CtIP Δ PLDLS. Cells were harvested 40 hrs post-transfection and lysed in IP buffer. Ten percent of the extract was loaded in input lanes 1 and 2. Gal4 DNA binding domain antibody (Santa Cruz, monoclonal) was used to immunoprecipitate Gal4-CtBP. Myc antibody (Santa Cruz, 9E10) was used in Western blotting to detect CtIP in the immunoprecipitates (lanes 3 and 4). The blot was stripped and re-probed with Gal4 DNA binding domain antibody to verify that equal amounts of Gal4-CtBP were immunoprecipitated (lanes 3 and 4).
- B. C33A cells were transiently transfected with 1 μ g β -gal, 0.5 μ g of the pSVECG reporter and 2 μ g of Gal4-CtIP or Gal4-CtIP Δ PLDLS. Western blotting was performed to verify that transfected constructs expressed equal protein. As controls, C33A cells were transfected with 1 μ g β -gal, 0.5 μ g pSVECG reporter and 2 μ g of control vector. Cells were harvested 40 hrs post-infection and CAT activity was assayed. β -Gal values were used to normalize for transfection efficiency. Results of typical experiments are shown.
- C. Same as in B except that cells were transfected with 1 μ g β -gal, 0.5 μ g pSVECG reporter, and 2 μ g of Gal4-CtBP or 20 μ g of Gal4 CtIP.

A.



B.



C.

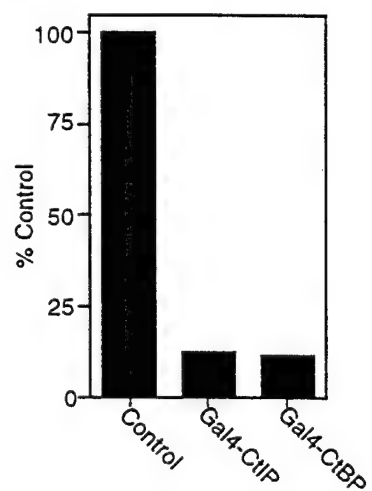
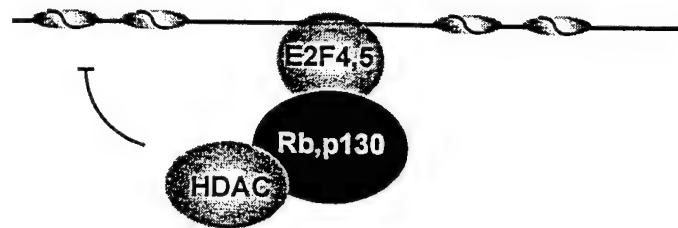


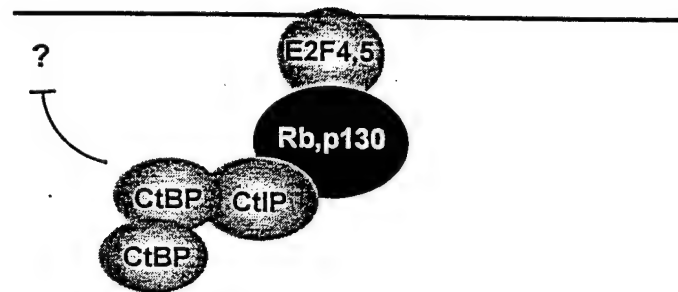
Figure 5. Alternative Mechanisms for Rb/p130-Mediated Repression

E2F target promoters can be repressed in two fashions. Histone deacetylase is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. Histone deacetylase then modifies the histones proximal to the promoter causing transcriptional silencing. Rb and p130 recruit CtIP/CtBP to E2F complexes. CtIP bridges the interaction between CtBP and the E2F/Rb complex. CtBP, most likely acting as a dimer, then functions by an undetermined mechanism to mediate repression.

Recruitment of Histone Deacetylase



Recruitment of CtBP



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